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THE DISTRIBUTION OF ZINC, CADMIUM, LEAD AND COPPER WITHIN THE HEPATOPANCREAS OF A WOODLOUSE

Key words: Isopoda, hepatopancreas, heavy metals, X-ray microanalysis

ABSTRACT. The distribution of metals within the hepatopancreas of *Oniscus asellus* (Crustacea, Isopoda) from two uncontaminated sites, and two sites contaminated with zinc, cadmium and lead, has been studied by atomic absorption spectrophotometry, light microscopy, transmission and scanning electron microscopy and X-ray microanalysis. The hepatopancreas contains two types of intracellular granule. The first type, in the S cells, are spherical granules which contain copper, sulphur and calcium. In woodlice from contaminated sites, these 'copper' granules, also contain zinc, cadmium and lead. The second type, in the B cells, are flocculent deposits which contain iron. In woodlice from contaminated sites, these 'iron' granules also contain zinc and lead. Moribund woodlice from contaminated sites have large numbers of 'copper' and 'iron' granules in the hepatopancreas and a fine deposit of zinc and lead on the membranes of the cells. There are numerous microorganisms in close association with the microvillous border of the hepatopancreas of woodlice from all four sites. Within the microorganisms of *Oniscus asellus* from contaminated sites, there are deposits of material which contain zinc, lead, calcium and phosphorus 'Copper' and 'iron' granules could have evolved as storage sites for essential metals to be utilized when demand from the body exceeds uptake from the food. Woodlice in contaminated sites may be able to 'detoxify' potentially harmful amounts of essential and non-essential metals by storing them in a relatively insoluble form within these granules.

Introduction

WORK on the distribution of heavy metals in the tissues of terrestrial isopods has shown that the hepatopancreas is by far the most important storage organ of zinc, cadmium, lead, and copper (Coughtrey *et al.*, 1977; Hopkin and Martin, 1982; Wieser, 1979). Indeed, specimens of *Oniscus asellus* from sites contaminated with these metals may contain concentrations of zinc, cadmium, lead and copper in the hepatopancreas of about 1%, 0·5%, 2·5% and 3% of the dry weight respectively, which are among the highest so far recorded in the soft tissues of any animal (Hopkin and Martin, 1982).

Studies on the fine structure of the hepatopancreas of crustaceans have shown that the cells may contain several types of granular inclusion (for a review of this work see Icely and Nott, 1980). In terrestrial isopods, two main types have been observed, The first consist of spherical accumulations of homogeneous electron-dense material which contain copper. These 'copper' granules occur with the S cells of the hepatopancreas (Alikhan, 1972; Wieser, 1968; Wieser and Klima, 1969). The second type consist of more loosely bound deposits of flocculent material which contain iron (Hryniewiecka-Szyfter, 1972, 1973). These 'iron' granules are stored within the B cells of the hepatopancreas.

In this paper, the concentrations of zinc,

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cadmium, lead and copper in the four tubules of the hepatopancreas of *Oniscus asellus* from a contaminated site have been measured. The ultrastructure of the hepatopancreas of woodlice from two uncontaminated and two contaminated sites has been studied by light, transmission and scanning electron microscopy, and the elemental composition of intracellular granules determined by X-ray microanalysis.

Materials and Methods

Samples of mature *Oniscus asellus* were collected from Trelawne Wood (British Ordnance Survey Grid Reference SX 224 546), Midger Wood (ST 796 893), Hallen Wood (ST 555 802) and from rough grassland near the village of Shipham (ST 452 572) during April 1981. Trelawne and Midger are uncontaminated sites whereas Hallen (near a smelting works) and Shipham (disused zinc mine) are heavily contaminated with zinc, cadmium and lead (Hopkin and Martin, 1982). Each population was maintained for about six months at room temperature in separate plastic tanks containing leaf litter from the collection sites. During this period, all the woodlice in the cultures from Trelawne and Midger remained vigorous and apparently healthy. However, many of the woodlice in the cultures from Hallen and Shipham became immobile and would move their limbs only weakly when stimulated. The hepatopancreas of these animals was grey or white instead of the usual yellow/brown colour and the tubules were deformed.

Atomic absorption spectrophotometry

The distribution of zinc, cadmium, lead and copper between the four tubules of the hepatopancreas of five moribund and five healthy woodlice from Shipham was determined. The tubules and the hindgut were removed from each individual and placed on small (5 mm^2) pieces of Millepore filter paper which had been dried and weighed.

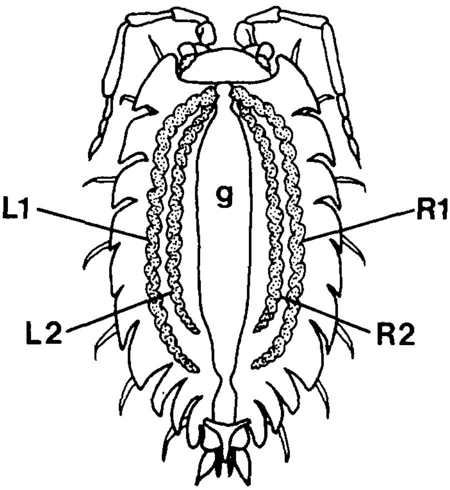


Fig. 1. Schematic dorsal view of the digestive organs of *Oniscus asellus* showing the position of the hindgut (g) and the right (R1, R2) and left (L1, L2) tubules of the hepatopancreas. × 10 approx.

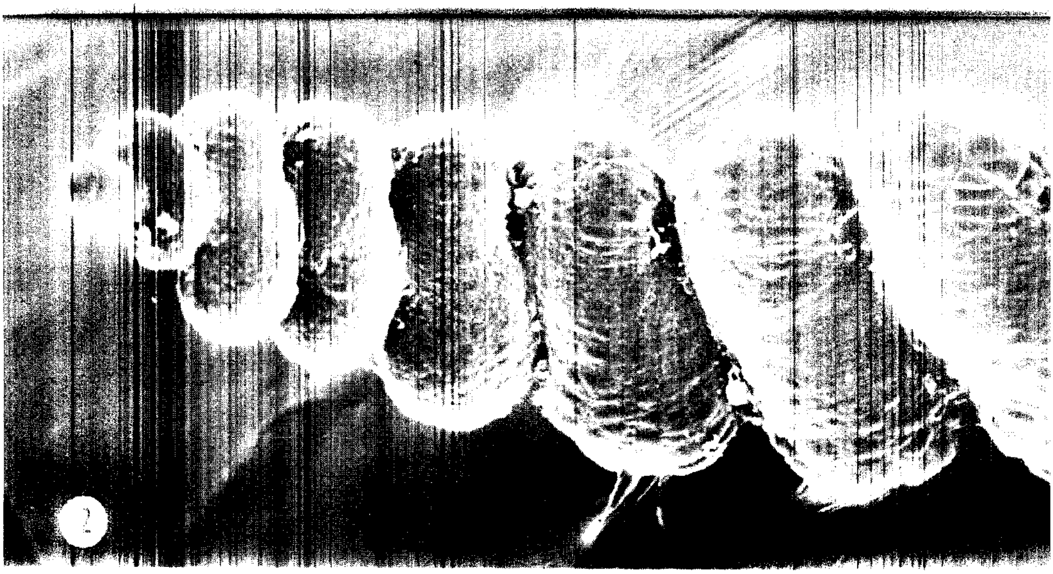
Fig. 2. A single tubule from the hepatopancreas of *Oniscus asellus*. The pronounced spiral groove which increases the internal and external surface area of the gland is always present. SEM × 190.

Fig. 3. Network of circular and longitudinal muscles surrounding a single tubule. SEM × 230.

Fig. 4. Transversely fractured tubule. A more detailed view of a fractured surface is shown in Fig. 8. SEM × 135.

Fig. 5. Transverse section through a single tubule of a healthy woodlouse from Hallen. The B cells (B) are larger than the S cells (S) and project into the lumen (lum). LM × 125.

Fig. 6. Longitudinally fractured tubule showing the regular distribution of the B cells which project into the lumen. SEM × 360.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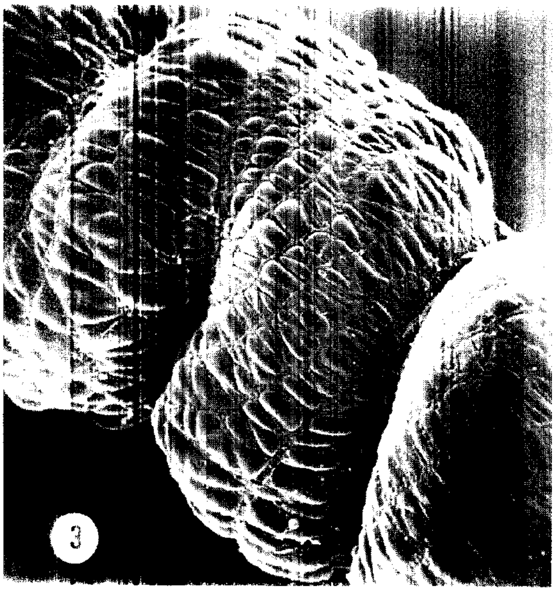
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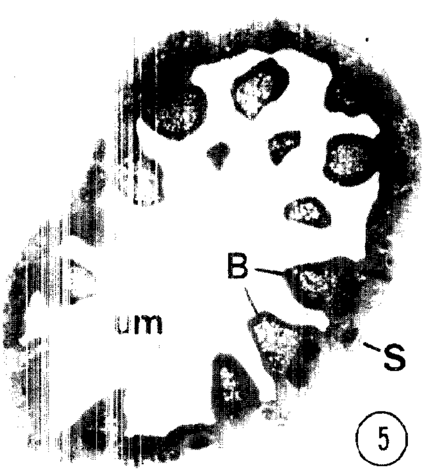
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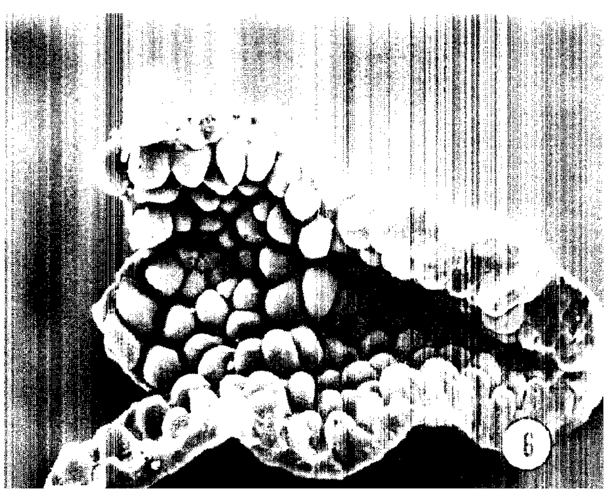
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These, together with the rest of the body including the exoskeleton (rest) were placed in glass Petri dishes and dried overnight at 70°C. After cooling to room temperature, the samples were weighed on a microbalance, To prevent fluctuations in the moisture content of the tissues, a few crystals of silica gel were placed in each Petri dish and the weighing chamber of the microbalance.

The samples were digested in boiling concentrated Aristar grade nitric acid (BDH Chemicals, Poole, Dorset, U.K.) and diluted to 5 ml with de-ionized distilled water. The digests were analysed for zinc, cadmium, lead and copper by flame (Varian AA775) or flameless (Varian AA6 and CRA 90) atomic absorption spectrophotometry. In all cases, correction for non-atomic absorption was made automatically with a deuterium or hydrogen lamp respectively (for further details see Hopkin and Martin, 1982).

Transmission electron microscopy (TEM) and X-ray microanalysis

Tubules were fixed in 2·4% glutaraldehyde in 0·1 M cacodylate buffer for 2 hr at room temperature and washed for 1 hr in 0·1 M cacodylate buffer. After dehydration through a graded series of ethanols at room temperature, the material was embedded in Spurr's low viscosity epoxy resin. Sections of up to 1 μm in thickness were cut on to water with an LKB Ultrotome III, picked up on uncoated aluminium grids and examined in a Philips 300 TEM operating at an accelerating voltage of 60, 80 or 100 kV. Areas of the sections were analysed with an Edax energy-dispersive X-ray microanalyser using the STEM attachment of a Philips 301 TEM, or a Kevex energy-dispersive X-ray detector with LINK analyser on a JEOL 100S TEM. Grids were held in a graphite specimen holder in all cases to reduce background radiation.

Unstained sections can be difficult to interpret in the TEM due to poor contrast. However, they have two main advantages over material which has been 'conventionally' prepared by post-fixation in osmium and staining with uranium and lead. Firstly, naturally occurring deposits of metals can be located rapidly in the tissues, Secondly, the electron beam can penetrate much further through the specimen and sections of up to 1 μm in thickness can be examined.

Scanning electron microscopy (SEM)

Preparation was the same as that for TEM until final dehydration when the tissue was transferred from absolute ethanol to acetone. After 30 min, the material was frozen and fractured under liquid nitrogen and the fragments returned to acetone at room temperature. The tissue was critical-point-dried or air dried, mounted on aluminium stubs and coated with gold in a DC sputter coater before examination in a Cambridge Instruments S4, or Philips 501B SEM.

Light microscopy (LM)

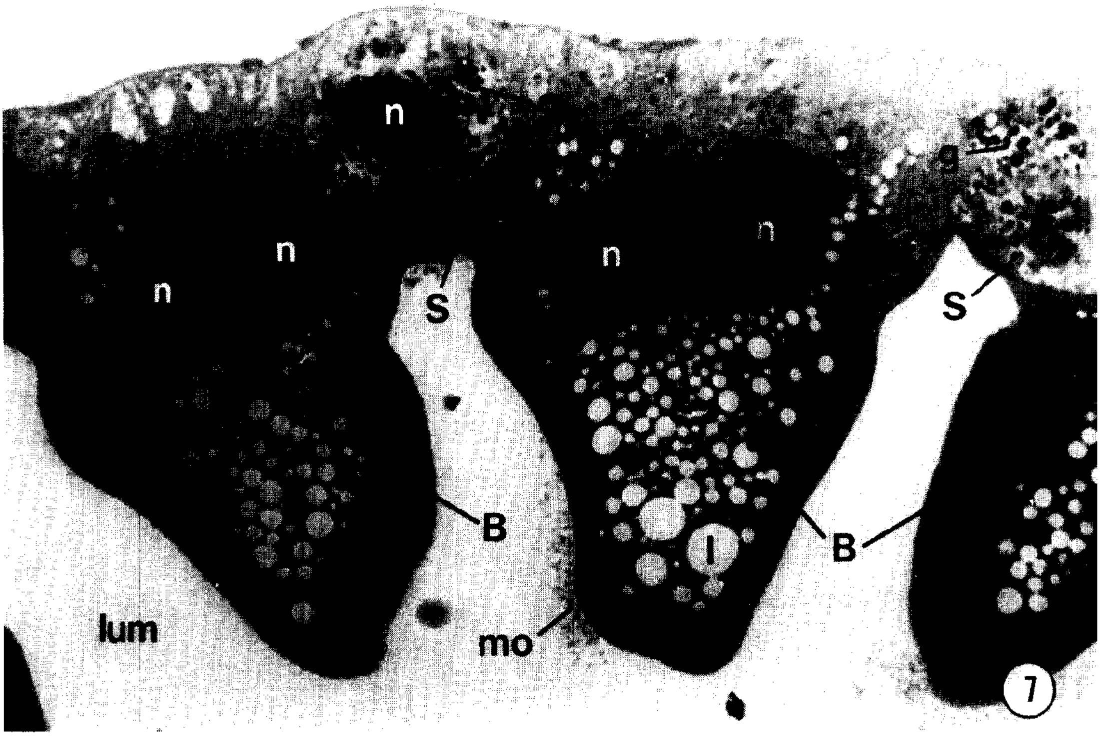
Sections of 1 μm in thickness were cut with an LKB Ultrotome III and stained with an aqueous solution of 1% toluidine blue and 1% borax.

Results

The hepatopancreas of *Oniscus asellus*

Fig. 7. B and S cells shown in Fig. 5. There are spaces (l) in the B cells (B) left by lipid droplets dissolved out during preparation. The S cells (S) contain numerous 'copper' granules (g). There are microorganisms (mo) in close association with the microvillous border (see also Figs. 14, 15). n, nucleus; lum, lumen. LM ×720.

Fig. 8. Transversely fractured tubule of a healthy woodlouse from Hallen. The 'copper' granules (g) are prominent within the S cells (S). These granules are shown in greater detail in Fig. 9. The spaces (l) in the B cells (B) are left by lipid droplets dissolved out during preparation, lum. lumen. SEM ×1600.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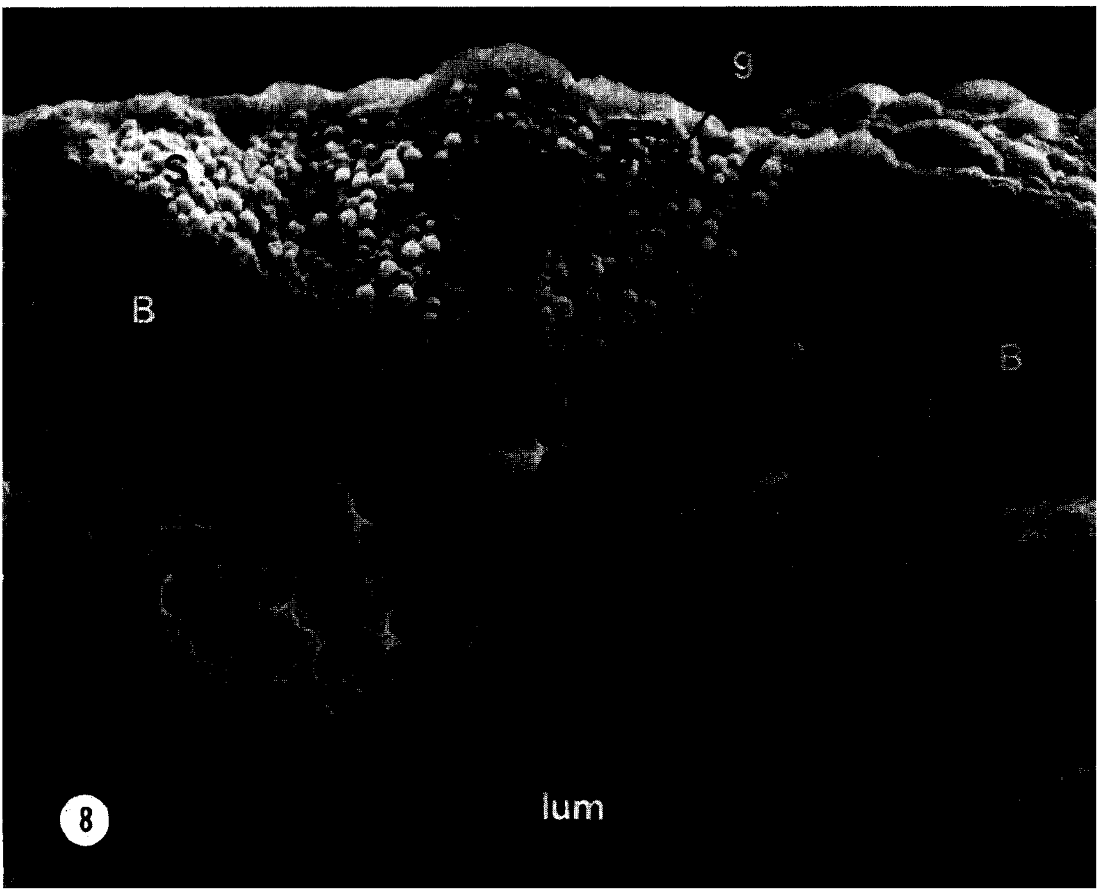
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consists of four blind-ending tubules which open into the anterior end of the hindgut (Fig. 1). The gland performs three main functions. It secretes digestive enzymes (Hartenstein, 1964), is the main site of food absorption and is the main store of metabolic reserves (Szyfter, 1966).

Each tubule is surrounded by a network of circular and longitudinal muscles (Figs. 2, 3). When the muscles contract, the diameter of the tubules is reduced and material is squeezed from the lumen of the gland into the hindgut. It is assumed that when these muscles relax, material flows in the reverse direction. The pronounced spiral groove on each tubule (Fig. 2) increases the internal and external surface area of the hepatopancreas.

There are considerable differences in concentrations of zinc, cadmium, lead and copper between the whole hepatopancreases of woodlice from Shipham. However, within individual animals, the concentrations of metals in each tubule are the same (Table 1). The levels of zinc, cadmium and lead are consistently higher in the hepatopancreas of moribund animals than in those of healthy woodlice.

The cytology of the epithelial cells in the hepatopancreas of isopod crustaceans has been studied by a number of workers (e.g. Alikhan, 1972; Brown, 1978; Donadey and Besse, 1972; Hryniewiecka-Szyfter, 1972, 1973; Licar, 1975; Patanè, 1934; Szyfter, 1966; Vernon *et al.*, 1974; Wieser and Klima, 1969) and in *Oniscus asellus* in particular by Clifford and Witkus (1971). Thus, only a brief description of the epithelium will be given here.

There are two types of cells which are distinguished readily in light micrographs (Figs. 5, 7). The B cells are large and project into the lumen of the hepatopancreas (Figs. 4–8). Among the numerous lipid droplets in the B cells, there are deposits of flocculent material (Figs. 10–12, 15). In woodlice from Trelawne and Midger, X-ray spectra of the deposits always have a large peak for iron and occasionally, peaks for phosphorus, sulphur and calcium (Fig. 17). In animals from Hallen and Shipham, these 'iron' granules also contain zinc and lead (Fig. 18).

The S cells are much smaller than the B cells (Figs. 7, 8). Throughout the cytoplasm of the S cells of woodlice from Trelawne and Midger, there are spherical granules. X-ray spectra of these inclusions have prominent peaks for copper, sulphur and calcium and occasionally, small peaks for phosphorus and iron (Fig. 19). In animals from Hallen and Shipham, these 'copper' granules also contain cadmium, zinc and lead (Figs. 9, 13, 20, 21).

In moribund woodlice from Hallen and Shipham, there are large numbers of 'iron' and 'copper' granules in the B and S cells, respectively (Figs. 10, 11). In addition, the membranes of the cells are lined with a fine deposit of electron-dense material

Fig. 9. 'Copper' granules within an S cell. SEM × 17,000.

Fig. 10. Longitudinal section through a single tubule of the hepatopancreas of a moribund animal from Hallen. There are extensive deposits of graular material in the B cells (B) and S cells (S). LM × 175.

Fig. 11. B and S cells (labelled in Fig. 10) packed with 'iron' (Fe) and 'copper' (g) granules. n, nucleus; lum, lumen. LM × 720.

Fig. 12. Border between an S and B cell in the hepatopancreas of a moribund *Oniscus asellus* from Shipham. The intercellular membranes are lined with electron-dense material (m) which contains zinc and lead. The 'iron' granules (Fe) in the B cell contain iron, zinc and lead. The 'copper' granule (g) in the S cell contains zinc, cadmium, lead and copper. TEM × 17,000.

Fig. 13. Intact 'copper' granule within an S cell. Many of these granules are damaged or torn out during sectioning. TEM × 34,000.


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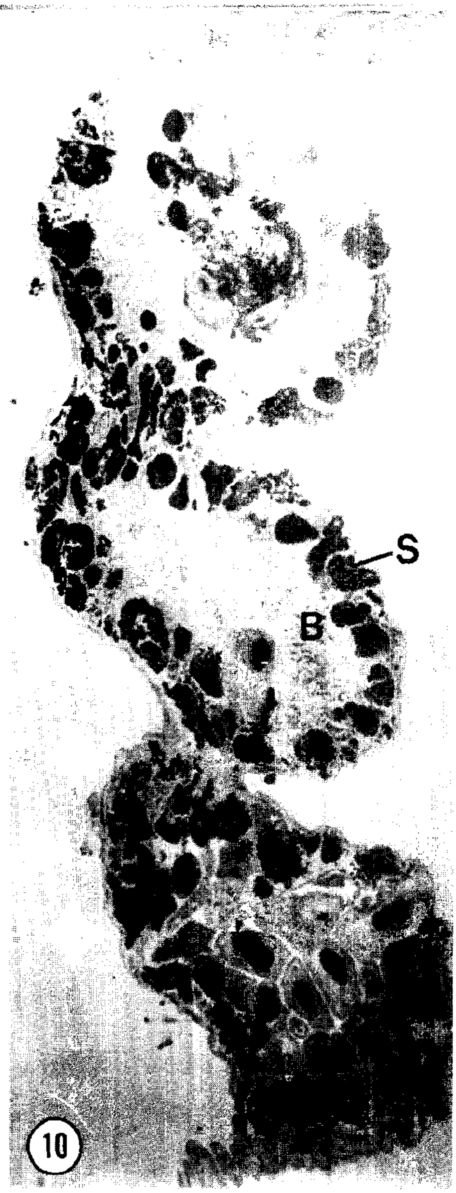
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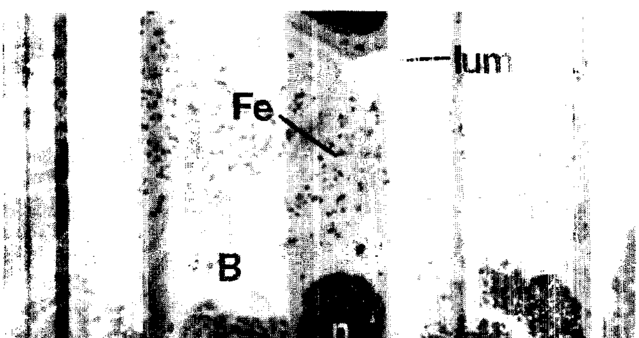
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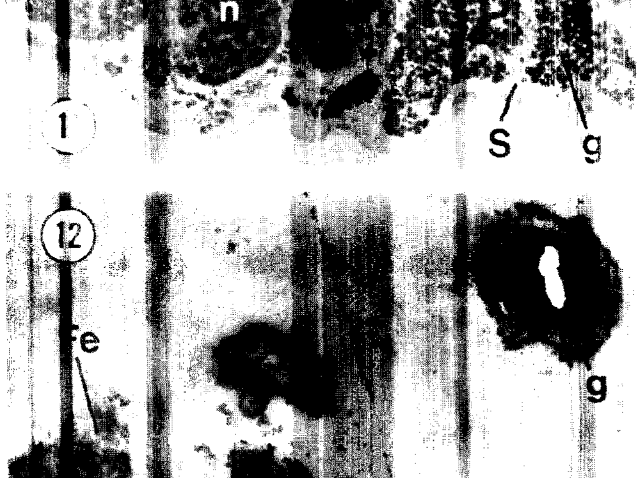
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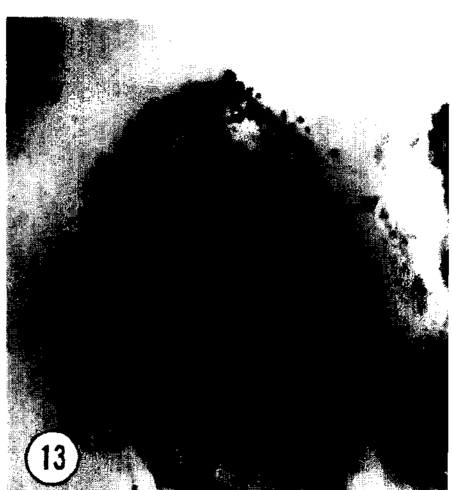
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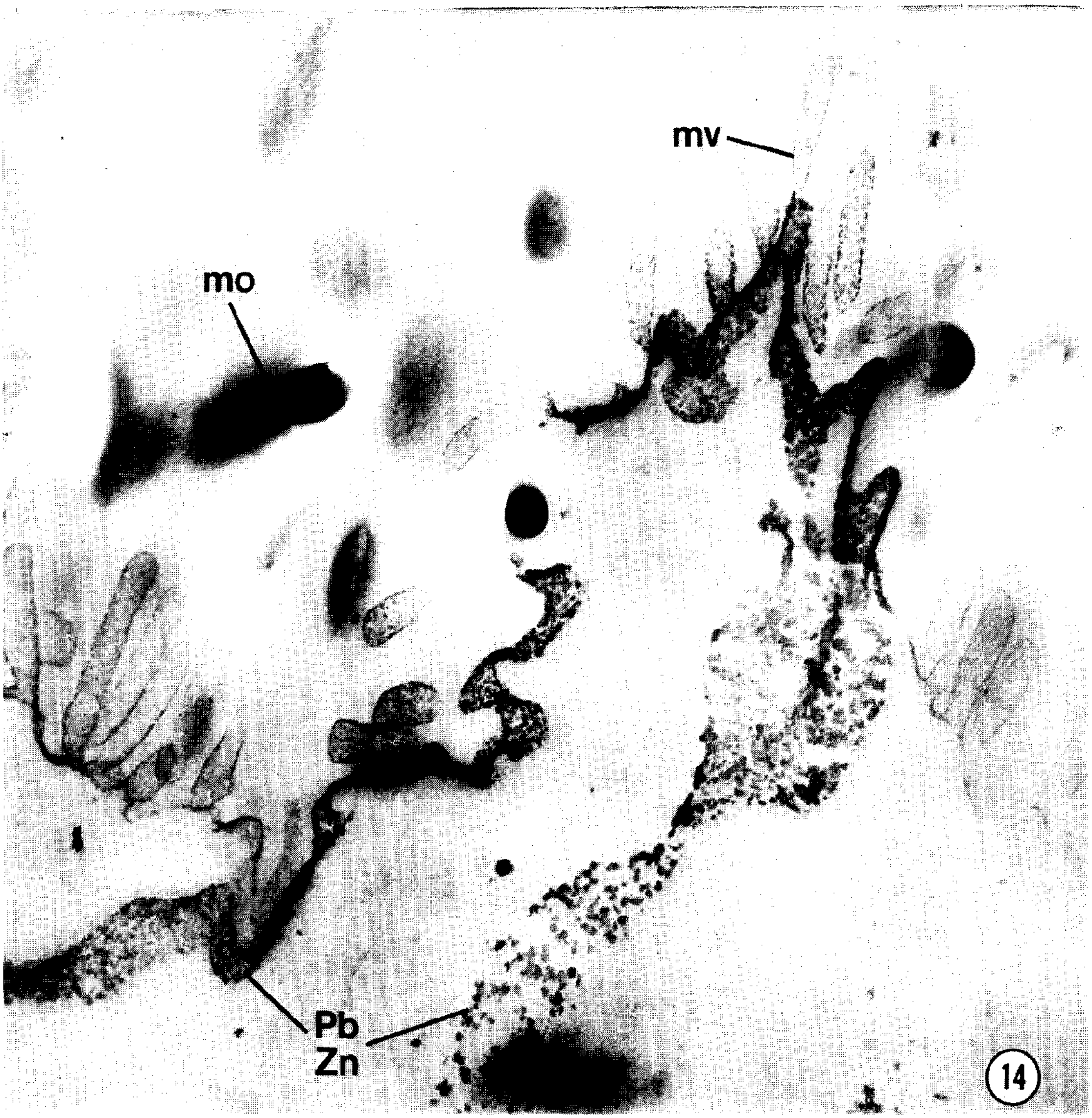
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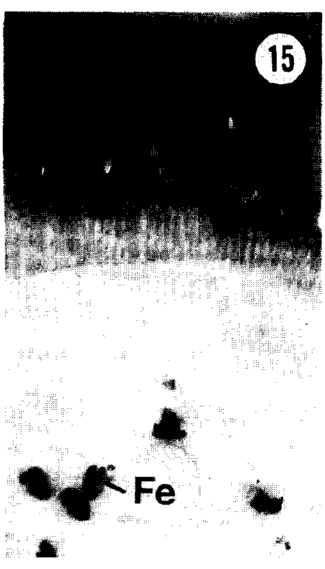
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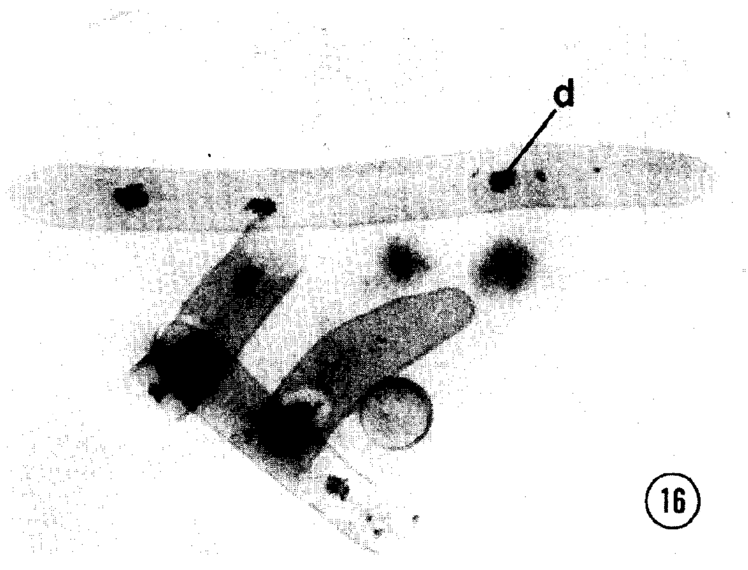
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Table 1. *Mean dry weight (mg ± SE) and mean concentrations of metals (μg g⁻¹ dry weight ± SE) in the four tubules of the hepatopancreas, the hindgut amd the rest of the body tissues of healthy and moribund* Oniscus asellus *from Shipham, a disused zinc mine* ($n = 10$)

Tissue	Dry weight	Zn	Cd	Pb	Cu
Tubule L1	0·349 ± 0·029	14,472 ± 2148	4125 ± 514	5273 ± 666	1086 ± 221
Tubule L2	0·212 ± 0·014	15,550 ± 2258	4673 ± 497	5661 ± 943	1481 ± 278
Tubule R1	0·212 ± 0·015	16,100 ± 2016	4773 ± 421	5469 ± 787	1373 ± 228
Tubule R2	0·335 ± 0·040	14,787 ± 1689	4282 ± 407	5429 ± 804	1221 ± 227
Total hepatopancreas	1·108 ± 0·088	14,939 ± 1894	4350 ± 424	5365 ± 740	1239 ± 221
Range hepatopancreas		(8036–27,753)	(3113–7753)	(1960–8032)	(417–2434)
Hindgut	1·871 ± 0·270	852 ± 114	72·5 ± 16·7	508 ± 29	87·0 ± 2·9
Rest	19·489 ± 1·553	77·4 ± 4·9	4·24 ± 0·74	18·5 ± 2·8	59·1 ± 3·0
Whole animal	22·467 ± 1·595	830 ± 56	217 ± 14	328 ± 46·2	116 ± 12

The differences between the concentrations of each metal in whole hepatopancreases (range) are statistically significant (ANOVA $P < 0{\cdot}01$). However, the small differences between the mean concentrations of metals in the four tubules are not significant for zinc, cadmium and lead (ANOVA $P > 0{\cdot}05$), and are only marginally significant for copper (ANOVA $P < 0{\cdot}05$). The differences in the mean concentrations of copper between left and right pairs of tubules are not significant ($P > 0{\cdot}05$).

(Figs. 12, 14) in which zinc and lead are detected by X-ray microanalysis.

In specimens of *Oniscus asellus* from all four sites, there are numerous microorganisms in close association with the microvillous border of the hepatopancreas (Fig. 15). In woodlice from Hallen and Shipham, the microorganisms contain deposits of material (Fig. 16) in which zinc, lead, calcium and phosphorus are detected by X-ray microanalysis (Fig. 22). Microorganisms in the hepatopancreas of woodlice from Trelawne and Midger do not contain this material.

Discussion

Previous work has shown that there is little difference in the amounts of copper in the left and right pairs of tubules of the hepatopancreas of individual woodlice (Wieser and Klima, 1969), even though the concentrations in the whole organ may differ substantially between individuals from the same site (Alikhan, 1972) due presumably, to variations in the stage of the moult cycle and choice of food. Analysis of woodlice from Shipham (Table 1) confirm these findings and show that the four tubules of the hepatopancreas are of equal importance in the storage of zinc, cadmium and lead.

In *Oniscus asellus*, the hepatopancreas may act as a 'barrier' to the diffusion of potentially harmful amounts of essential and non-essential metals into the blood by

Fig. 14. Microvillous borders of B and S cells in the hepatopancreas of a moribund *Oniscus asellus* from Hallen. The section is 1 μm in thickness. Deposits of material which contain lead and zinc (Pb, Zn) are present on the membranes and the microvilli (mv). mo, microorganisms; TEM ×62,000.

Fig. 15. 'Iron' granules (Fe) in the apical region of a B cell of a woodlouse from Shipham. There are numerous microorganisms (mo) in close association with the microvillous border. TEM ×6000.

Fig. 16. Detail of microorganisms shown in Fig. 15. The deposits of material (d) contain zinc, lead, calcium and phosphorous (see Fig. 22). TEM ×53,000.

precipitation on to 'copper' and 'iron' granules as the elements enter the S and B cells. In addition, metals which enter the blood from the hindgut could be removed across the basement membrane of the hepatopancreas and stored in a similar manner. In this way, the hepatopancreas may regulate the concentrations of metals in the blood. Indeed, it has been shown that the mean concentrations of zinc, cadmium, lead and copper in the 'rest' fraction of *Oniscus asellus* from a range of contaminated sites are always less than about 70, 5, 65 and 65 $\mu g\ g^{-1}$ dry weight respectively, even though the levels in the hepatopancreas reach several thousand $\mu g\ g^{-1}$ dry weight (Hopkin and Martin, 1982).

In woodlice from uncontaminated sites such as Trelawne and Midger, 'copper' and 'iron' granules could have evolved as storage sites for essential metals to be utilized when demand from the body exceeds uptake from the food. Thus, woodlice may be 'pre-adapted' to living in moderately contaminated sites since they are able to store potentially harmful amounts of metals in a relatively insoluble form in 'copper' and 'iron' granules. Such a system may require less energy than would be needed to maintain a concentration gradient between the cytoplasm of the cells of the hepatopancreas and the digestive fluid (see Simkiss, 1976, 1977 for further discussion of this concept). However, at sites which are heavily contaminated with metals such as Hallen and Shipham, the capacity of the hepatopancreas to 'detoxify' these elements may become saturated. If this were to occur, the concentrations of metals in the blood would increase, essential biochemical reactions within the body tissues would be disrupted and the animal may die. Moribund woodlice from Hallen and Shipham may have been affected in such a way.

Animals kept in laboratory cultures are probably more susceptible to poisoning by heavy metals than those in the field because they have greater access to a moist faecal substrate in which the metals may be made more 'available' by microorganisms (White, 1968; Wieser *et al.*, 1977). However, some specimens of *Oniscus asellus* from Hallen and Shipham, collected and dissected

Fig. 17. Elemental spectrum of an 'iron' granule in a B cell of *Oniscus asellus* from Midger (uncontaminated site). Iron (6·4 keV) is always detected in this material. Peaks for phosphorus (2·0 keV), sulphur (2·3 keV) and calcium (3·7 keV) are occasionally present. The peaks for aluminium (1·5 keV) and chlorine (2·6 keV) in this, and in subsequent spectra, are derived from the spectrum support grid and the resin respectively.

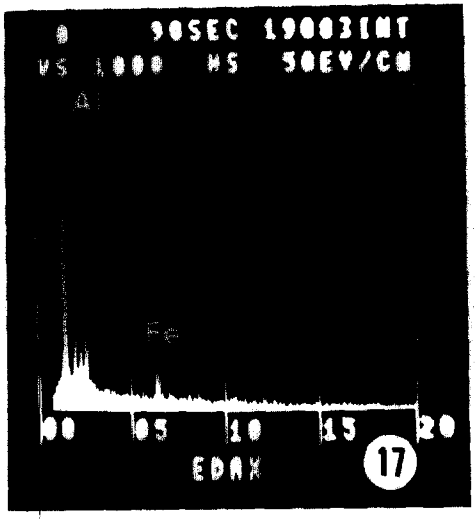
Fig. 18. Elemental spectrum of an 'iron' granule in a B cell of *Oniscus asellus* from Hallen (contaminated site). The main elements detected are iron, zinc (8·6 keV, 9·6 keV) and lead (2·3 keV, 10·5 keV, 12·6 keV, 14·8 keV). Phosphorus, calcium and very occasionally copper (8·0 keV) may be detected also. The $M\alpha$ peak for lead at 2·3 keV overlaps the $K\alpha$ peak for sulphur at 2·3 keV. Thus, the presence or absence of sulphur from granules containing lead cannot be confirmed.

Fig. 19. Elemental spectrum of a 'copper' granule in an S cell of *Oniscus asellus* from Midger. Copper (0·9 keV, 8·0 keV, 8·9 keV), sulphur and calcium are always detected. Small peaks for phosphorus and iron are occasionally present.

Fig. 20. Elemental spectrum of a 'copper' granule in an S cell of *Oniscus asellus* from Hallen. The main elements detected are cadmium (3·1 keV, 3·3 keV), calcium copper, zinc and lead. Phosphorus and iron may be detected also.

Fig. 21. The presence of cadmium in the 'copper' granules is confirmed by the peaks at 23·1 keV and 26·0 keV. This spectrum is from the same granule analysed in Fig. 20.

Fig. 22. Elemental spectrum of deposits of material within microorganisms shown in Fig. 16. The main elements detected are zinc, lead, calcium and phosphorus.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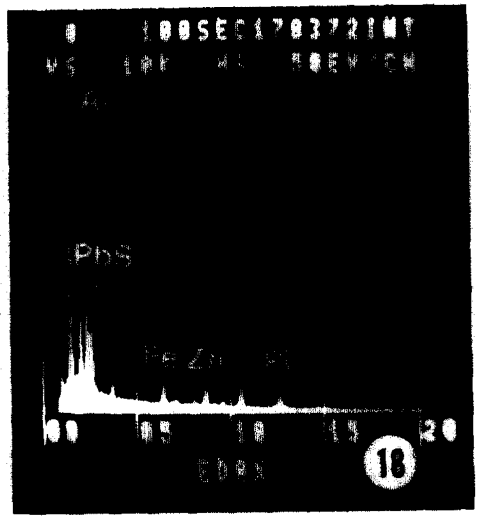
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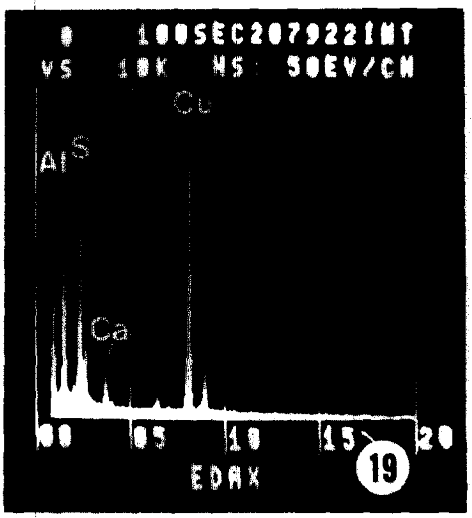
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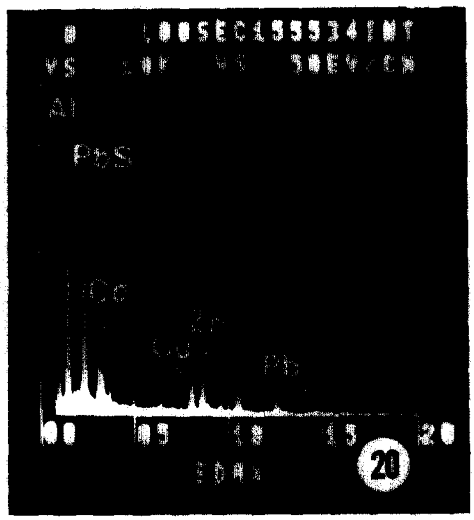
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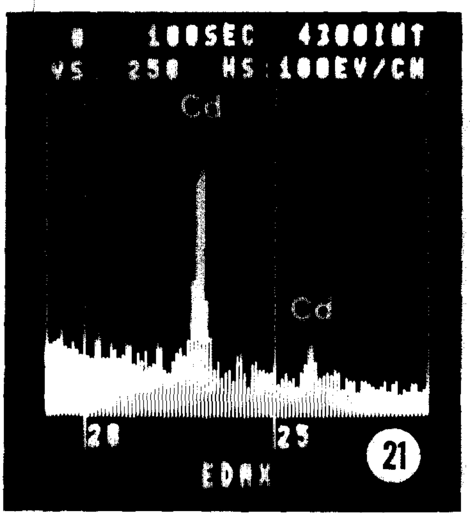
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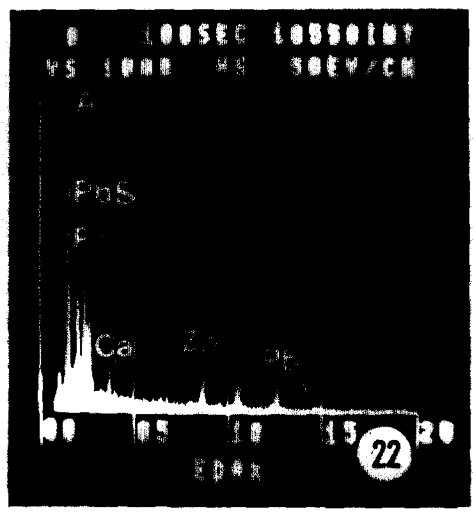
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on the same day, have a hepatopancreas of similar appearance to that of moribund animals from the laboratory cultures. Therefore, the high levels of metals in the leaf litter at these contaminated sites are probably adversely affecting *Oniscus asellus* in the wild also.

The microorganisms in close association with the microvillous border of the hepatopancreas of *Oniscus asellus* are of similar appearance to the bacterial flora in the lumen of this gland in *Porcellio dilitatus* (Donadey and Besse, 1972). *Philoscia muscorum* is thought to utilize cellulase secreted by microorganisms in the gut (Hassall and Jennings, 1975) and it is possible that the microflora in the hepatopancreas of *Oniscus asellus* performs a similar function.

The tolerance of the gut microflora to zinc, cadmium and lead increases when *Oniscus asellus* feeds on litter contaminated with these metals (Coughtrey *et al.*, 1980). One way of achieving tolerance would be to 'detoxify' metals which cross the cell wall by storing them as insoluble deposits in the cytoplasm (Fig. 16). Indeed, bacteria grown on a lead-rich medium absorb the metal until it comprises 36% of the dry weight of the organisms (Aickin and Dean, 1977).

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